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Molecular and Phytochemical Characterization of Some Silybum Landraces Grown in Egypt

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ABSTRACT

A total of 20 random primers were evaluated for their ability to prime PCR amplification of 12 Silybum genotypes in a preliminary survey using genomic DNA of plants from each genotype. RAPD analysis revealed 128 scorable bands from two primers, including 36 (28.125%) polymorphic bands. The band pattern revealed differences between the collected genotypes. Certain band changes were found in Romanin genotype plants and between Egyptian genotypes, suggesting the existence of genetic variation which might affect the biochemical synthesis of the different genotypes tested in this study. The results of HPLC analyses for the different genotypes, revealed that the highest content of silychristin, Silydianin, Silybinin (A, B) and total silymarin were detected in samples collected from Romanian genotype cultivated in Egypt, Desert road of Alexandria genotype, Kafr El-Sheikh genotype and Bani-sewaf genotype, respectively. The lowest content of silychristin, Silydianin, Silybinin (A, B) and total silymarin were found in samples collected from Desert road of Alexandria genotype where silychristin was absent, agricultural road of Alexandria genotype where Silydianin was absent, desert road of Alexandria genotype and Romania genotype, respectively. The results of this study confirmed that the differences in geographical locations and the genetic variation between silybum genotypes have a great effect in their RAPD fingerprints and contents of silymarin.

Key Words: Marianum, RAPD, HPLC, silymarin.

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INTRODUCTION

The Milk thistle *Silybum marianum* L. Gaertn belongs to Asteraceae family. It is an annual or biannual herbaceous plant that is widespread in temperate American countries, Australia and areas of Mediterranean climate. In Egypt it grows wild in most districts especially in Nile Delta. This plant has been widely used in traditional European medicine. Nowadays silymarin, the purified extract of the fruits and its main constituents, silybin and the isomers silydinin and silicristin and related compounds are used in the treatment of various liver diseases *(Heywood and Harborne 1977)*. Silymarin and similar compounds have proved to be quite safe clinically in liver patients with no hazardous side reactions and is a stable compound and in pure or highly purified from can be stored for long periods. The flavonoid silymarin and one its structural components, silibinin, have been well characterized as hepato-protective substances. However, little is known about the biochemical mechanisms of action of these substances. Recent investigations elucidated the molecular action of the flavonoid. It is suggested that the biochemical effects observed for the flavonoid in experimental models may settle the basis for understanding the pharmacological action of silymarin and silibinin *(Valenzuela and Garrido 1994)*. Botanicals have been used traditionally by herbalists and indigenous healers worldwide for the prevention and treatment of liver disease. Clinical research in this century has confirmed the efficacy of several plants in the treatment of liver disease. Basic scientific research has uncovered the mechanisms by which some plants afford their therapeutic effects. *Silybum marianum* (milk thistle) has been shown to have clinical applications in the treatment of toxic hepatitis, fatty liver, cirrhosis, ischemic injury, radiation toxicity and viral hepatitis via its antioxidative, anti-lipid peroxidative, antifibrotic, anti-inflammatory, immunomodulating and liver regenerating effects *(Luper 1998)*.

The PCR-based RAPD technique was applied and proved effective in a number of reports. RAPD assays utilize arbitrary 10-mer oligonucleotide sequences as primers *(Williams et al., 1990)*. Primers hybridise to two nearby sites in the template DNA that are complementary to the primer sequence. Deletions or insertions in the amplified regions or base changes altering primer binding sites will result in polymorphisms. RAPDs have proved to be very useful for the analysis of large number of genotypes *(Rafalski and Tingey 1993)*. RAPD analysis was used for detecting intra-clonal genetic variability in vegetatively propagated tea *(Singh et al., 2004)* and *in vitro* produced turmeric plants *(Salvi et al., 2001)*. RAPD analysis also used for assessment genetic variability of *in vitro* produced *Ocimum americanum* L plants *(Rady and Nazif 2005)*.

In this paper we have evaluated the technique of RAPD analysis for detecting polymorphism in different genotypes of silybum plants collected from various locations. Our specific objectives were to determine the potential for using this approach to generate polymorphic markers for genotype identification according to their silymarin content.

MATERIALS AND METHODS

Plant materials:
Seeds of *S. marianum* (12 genotypes) were collected from different geographical regions all over Egypt. Seeds were germinated on MS culture medium *(Murashige and Skoog 1962)* and incubated in a growth chamber at 25 ± 2°C under light conditions of 16 hr day photoperiod at intensity of 2000 Lux from cool white fluorescent lamps for about three weeks. Within 30 days of cultivation, the plantlets reached about 7 cm in height and used as starting plant material. DNA was extracted from leaves of different silybum genotypes using the cetyltrimethylammonium bromide (CTAB) method of *Doyle and Doyle (1990).*

Polymerase Chain Reaction (PCR):
PCR amplification for different isolated DNA was performed in 20 µl reaction mixture containing 40 ng genomic DNA, 0.5 unit Taq polymerase (Appliegene, Germany), 200 µM each of dATP, dCTP, dGTP, dTTP. 10 pmole random primer (20 different oligonucleotide were used) and appropriate amplification buffer. Out of 20 random primers screened, 2 primers produced clear reproducible bands (sequences presented in Table 1). The mixture was assembled on ice and amplification was performed for 45 cycles, using Perkin Elmer thermocycler (USA), as follows: One cycle at 92°C for 2 min. and then 44 cycles at 92°C for 30 sec., 35°C for 60 sec. and 72°C for 2 min. (for denaturation, annealing and extension, respectively). Reactions were finally incubated at 72°C for 10 min. All primers used were 10-mer random oligonucleotide sequences obtained from Operon Technologies Inc. (Alameda, CA, USA). The amplification products were analyzed by electrophoresis in 2% agarose in TBE (Tris-Borate-EDTA) buffer (pH 8.0), stained with 0.2 µg/ml ethidium bromide and photographed under UV light using red filter.

Phytochemical analysis:
Preparation of Samples for Chemical analysis (by HPLC):
About one gram of the prepared calluses or cells was homogenized with 15 ml of 80% (v/v) methanol. The homogenate was kept at -40°C for 48 h and then filtered and concentrated under reduced pressure at 40°C. The dry residue was resuspended in 3 ml of distilled water, extracted twice with 6 ml of pure ethyl acetate, filtered and desiccated under reduced pressure at 40°C. the residues were dissolved in Methanol HPLC grade and, an aliquot of 20 µl were injected into the HPLC column. and subjected for HPLC analyses using the following conditions:

Column: C_{18} (5 µm), 250 X 4.6 mm i.d.
Temperature: Ambient
Flow rate: 1.5 ml / min.
Detector: UV variable wavelength detector adjusted at 288 nm.
Injection volume: 20 µl.
Mobile Phase: Isocratic Mixture of Methanol: Water: Glacial acetic acid 40:60:5 v/v/v.

Preparation of standard solutions:
About 10 mg of the authentic samples of, silybinin, silychristin, silydianin and silymarin reference material produced by Madaus were accurately weighed, separately, in 10 ml measuring flasks and brought to volume with methanol HPLC grade.

RESULTS

RAPD analysis:
A total of 20 random primers were evaluated for their ability to prime PCR amplification of 12 Silybum genotypes in a preliminary survey using genomic DNA of one plant from each genotype. Only two primers amplified all DNA templates. Data of the RAPD fragments generated by the two random primers used in the analysis of all plants comprising this study are shown in (Table 1) and (Figure 1). These primers generated a total of 128 DNA fragments. Out of the 128 fragments, 36 (28.125%) were polymorphic. The number of scorable RAPD fragments generated per primer, with an average of 64 per primer and the number of polymorphic bands per primer, with an average of 18 polymorphic bands per primer. On the other hand, OPK2 primer gave the highest percentage of polymorphism 46.81, than the other primer OPK1, 17.28, (Table 1).

(Table 2) shows the presence and absence of polymorphic bands among leaves tissues of different Silybum genotypes seeds. OPK1 and OPK2 primers produced amplification products that were polymorphic among all genotypes. It could be noticed that, banding patterns produced by the two primers were located between 50 to 500 bp. The results indicated that the Romanin genotype exhibiting amplified products different than other Egyptian genotypes. However, most of the Egyptian genotypes gave polymorphic banding patterns.

Table 1: RAPD-PCR amplification products of leaves tissues of Silybum genotypes, using two primers.

Primer	Sequence 5'--------3'	Total number of bands	Number of polymorphic bands	Polymorphism (%)
OPK1	TGCCGAGCTG	81	14	17.28
OPK2	GTGAGGCGTC	47	22	46.81
Overall total		128	36	28.125

Table 2: Distribution and size of polymorphic bands in leaves tissues of Silybum, using two primers.

Primer	Size of polymorphic band (bp)	Distribution of polymorphic bands											
		Silybum cultivar											
		1	2	3	4	5	6	7	8	9	10	11	12
OPK1	400	-	+	-	-	-	-	-	-	-	+	-	-
	300	-	+	-	-	-	-	-	-	-	+	-	-
	250	-	-	-	-	-	-	-	+	-	-	-	-
	200	-	+	-	-	-	-	-	-	-	-	-	+
	100	-	-	-	-	-	-	-	+	+	-	-	-
	80	-	-	-	-	-	-	-	-	-	+	-	+
	50	-	-	-	-	-	-	+	-	+	+	+	-
OPK2	500	-	-	-	-	-	-	-	-	+	-	-	-
	450	-	-	-	-	-	-	-	-	+	-	-	-
	400	-	-	-	-	-	-	+	-		-	-	-
	350	-	-	-	-	-	-	-	-	+	-	-	-
	300	-	-	-	-	-	-	+	+		-	-	-
	200	-	-	-	-	-	+	+	+		-	-	-
	150	-	-	+	-	+	+	+	+	+	-	-	+
	120	-	-	+	-	-	-	-	-		-	-	+
	50	-	-	-	-	-	+	+	+	+	-	-	-

+ and - indicate the presence and absence of polymorphic band, respectively.

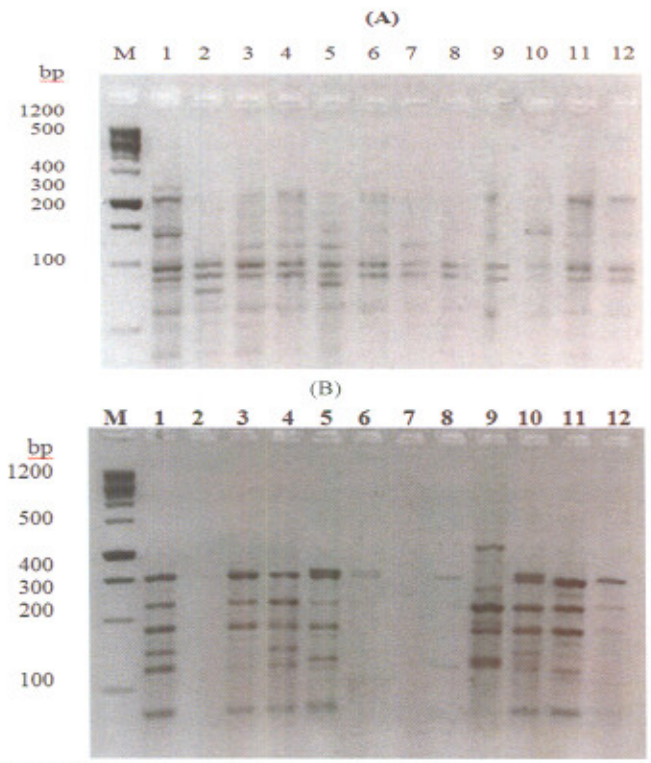

Figure 1: Agarose gel electrophoresis of RAPD fragments generated by primer OPK1 (A) and OPK2 (B) of different *S. marianum* genotypes.

Lane M indicates molecular weight DNA marker by bp.
Lane 1 = El-mansorya (red flower) Lane 2 = Kafr-El-Shiekh (red flower)
Lane 3 = Asuiet (red flower) Lane 4 = El-Menia (Samaloot) (red flower)
Lane 5 = Alexandria Agriculture road (White flower)
Lane 6 = Alexandria Desert road (Km 44) (red flower)
Llane 7 = Bani-Swaff (Violet flower)
Lane 8 = Alexandria Agriculture road (red flower)
Lane 9 = Romania cultivar Lane 10 = Romania cultivar grown in Egypt
Lane 11 = Interior oasis (Akhteem El- Kasr) (Violet flower)
Lane 12 = Interior oasis (Violet flower)

Phytochemical analysis:

Table 3: Illustrates the results of HPLC analyses of the methanolic extracts of samples of *S. marianum* collected from different localities in Egypt, the following are noticed: Samples which were collected from Desert road of Alexandria (44 Km), (6) and from Beni-suif governorate (7), all these samples showed relatively high content of silydianin, in the expense of that of silybinin since it reached (0.94 and 0.33 mg/g) represent 40.9 % and 10.71 % from the total flavonolignan, while silybinin (A, B) showed only (0.073 and 0.189 mg/g) represent 3.41% and 5.57 % of total flavonolignan, respectivly. While Silychristin was found to be in the normal range as that of standard sample of silymarin produced by Madaus, ca.20 % of total flavonolignan. In the same time sample collected from Kafr–El–Sheikh (2), showed very high content of Silybinin (SB), it reached up to 60 % of total silymarin (1.73 mg/g) with the normal range of silychristin content, 23.9 % (0.68 mg/g) with silydianin content of only 5% (0.14 mg/g). Also sample collected from agricultural road of Alexandria (5) with white flower, showed relatively high content of (SB.) 55.9% (1.12 mg/g) and Silychristin (SC.) 29% (0.58 mg/g) while complete absence of silydianin (SD.). Samples collected from El-Fayom (12), Agricultural road of Alexandria (violet flower) (8) and El Menia Governorate (4), the ratios of the SB. And SC. Were found to be the same as that found in the standard sample while silydianin (SD.) showed lesser content it reaches 3-5% (0.1, 0.1 and 0.11 mg/g), respectively.

In general, it could be concluded that; the highest content of silychristin was found in samples collected from Romanian genotype cultivated in Egypt (0.78 mg/g) followed by Interior oasis (Violet flower) (0.77 mg/g). The highest content of Silydianin was found in sample collected from Desert road of Alexandria 44 km (violet flower) (0.94 mg/g) followed by Beni–Suif (violet flower) (0.33 mg/g).The highest content of Silybinin (A, B) was found in sample collected from Kafr El-Sheikh (violet flower), (1.73 mg/g) followed by Agricultural road of Alexandria (white flower), (1.12 mg/g). The highest content of total silymarin was found in samples collected from Bani-sewaf (3.10 mg/g) followed by samples collected from Interior oasis (Violet flower) (2.96 mg/g). On the other hand, the lowest content of silychristin was found in samples collected from Desert road of Alexandria 44 km (violet flower) where silychristin was absent followed by Interior oasis (Akhteem El- Kasr) (Violet flower) (0.10 mg/g). The lowest content of silydianin was found in sample collected from Agricultural road of Alexandria (white flower) where Silydianin

was absent followed by Mansouria Giza (violet flower) (0.004 mg/g). The lowest content of Silybinin (A, B) was found in sample collected from Desert road of Alexandria 44 km (violet flower) (0.07 mg/g) followed by Romania cultivar (0.1 mg/g). The lowest content of total silymarin was found in samples collected from Romania cultivar (1.99 mg/g) followed by samples collected El-minia (violet flower) (2.01 mg/g).

Table 3: Comparative Studies of silymarin contents of different *Silybum marianum* L. samples collected from different areas by HPLC analyses (mg/g dry weight).

Sample	Silychristin	Silydianin	Silybinin (A,B)		Total Silymarin
			A	B	
1	0.41	0.004	0.22	0.34	2.34
2	0.68	0.14	0.65	1.08	2.85
3	0.73	0.05	0.31	0.62	2.29
4	0.49	0.11	0.33	0.54	2.01
5	0.582	-	0.71	0.41	2.51
6	-	0.94	0.023	0.05	2.31
7	0.65	0.33	0.05	0.139	3.10
8	0.53	0.1	0.59	0.48	2.41
9	0.15	0.12	0.05	0.05	1.99
10	0.78	0.13	0.14	0.29	2.36
11	0.10	0.17	0.11	0.2	2.41
12	0.77	0.1	0.5	0.8	2.96

1 = El-mansorya (red flower) 2 = Kafr-El-Shiekh (red flower)
3 = Asuiet (red flower) 4 = El-Menia (Samaloot) (red flower)
5 = Alexandria Agriculture road (White flower)
6 = Alexandria Desert road (Km 44) (red flower)
7 = Bani-Swaff (Violet flower)
8 = Alexandria Agriculture road (red flower)
9 = Romania cultivar
10 = Romania cultivar grown in Egypt
11 = Interior oasis (Akhteem El- Kasr) (Violet flower)
12 = Interior oasis (Violet flower)

DISCUSSION

RAPD (Random Amplified Polymorphic DNA) analysis: The analysis of genetic diversity plays an important role in breeding programs. In this order, molecular techniques could be used to evaluate diversity between ecotypes. The RAPD technique could be effective in detecting the genetic variation in silybum. The preliminary analysis of the variability indicators in silybum ecotypes, originating from the main cultivation areas, showed similar and dissimilar properties among them. From the obtained results, RAPD analysis demonstrated that DNA variations existed among the wild-types of silybum tested in this study. In this respect, many authors used molecular analysis for identification and surveying different cultivars grown in different locations. Comes and Abbott, *(2000)* were investigated RAPD and quantitative trait variation of the widespread and ephemeral *Senecio gallicus* were surveyed in 11 populations sampled from the Iberian Peninsula and southern France. Employing multivariate statistics, a moderate level of intraspecific differentiation was observed among populations from Iberian coastal and inland regions for both RAPDs and quantitative traits. However, RAPDs provided greater resolution in identifying additional population structure within the hypothesized, Pleistocene refugial source area of the species in coastal Iberia.

Also, RAPD markers were used to measure genetic diversity within and divergence among species of *Dendroseris* (Asteraceae: Lactuceae), *(Esselman et al., 2000)*. RAPD banding patterns distinguished all individuals examined and different mutilocus genotypes were found even in species exhibiting no allozyme diversity. RAPD band diversities ranged from 0.003 to 0.022 within species; >90% of total diversity was among species and <10% within them. Also, *Vilatersana, et al. (2005)* have clarified studies of the problem of the generic limits of Carthamus. They concluded that methodological problems posed by RAPD markers can be avoided by careful laboratory procedures and appropriate data analyses; they also suggest that this kind of marker is useful at low taxonomic levels and is, furthermore, complementary to DNA sequence analysis. Farag, et al. *(2006)* showed that with RAPD analysis no significant differences were observed among clove plants produced from beads soaked in calcium chloride solution for different periods whereas, in calli tissues grown on media containing mannitol, high percentage of polymorphisms were detected. These results suggest the existence of genetic variation which might affect the biochemical synthesis of plants derived from clove tissue culture. Also, *Jayaram and Prasad (2008)* reported that, RAPD markers were used to assess genetic diversity in *Oroxylum indicum* (L.) Vent (Bignoniaceae) a vulnerable medicinal plant collected from eight locations in Andhra Pradesh, India. High level of genetic similarity was observed in the collected accessions. Forty random primers, each with 10 bases generated a total of 188 polymorphic bands out of the 387 total bands, that is, polymorphism of 49.61% was observed. Overall genetic similarity based on 40 random primers was 87%. The results show that the genetic diversity of this species is low, possibly depicting a difficulty in adapting to environmental variations. Recently, *Bharmauria, et al. (2009)* found genetic variation in *Urtica dioica* plant samples, Examination of RAPD markers from six plant samples collected at different heights from sea level indicated that genetic variation was appreciable, as samples from lower altitudes showed low genetic similarity with samples collected from higher altitudes. Eight random decamer primers were used for RAPD amplification. The RAPD products ranged from 500 - 2800 bp. A total number of 134 RAPD markers were obtained from 8 primers, out of which 27 were polymorphic (20.2%) and the rest were monomorphic (79.8%). The polymorphism ranged from 4-50%. More recently, Abdoli, et al. *(2009)* indicated that, garlic is a valuable medicinal plant with variability in desirable morphological and physiological characteristics. Results indicated that five out of ten pair primers had no amplification. The analysis of genetic diversity plays an important role in breeding programs. A total of 35 RAPD bands were produced, 31 of which (88.5%) were polymorphic. In conclusion no significant relationship between genetic diversity detected by RAPD technique and geographical origins.

Phytochemical analysis: HPLC (high performance liquid chromatographic) with UV/VIS multiwavelength detector was used since all phenolic compounds show intense absorption in the UV region of the spectrum. The present method is simple, easy to use and effective enough for identification and quantification of major phenolic compounds in

aromatic and medicinal plants. A similar technique has been reported by other authors for the analysis of major flavonoid aglycones. Wallace, et al. *(2002)* were developed a HPLC analytical protocol for the separation of taxifolin, silychristin, silydianin, silybinin A, silybinin B, isosilybinin A and isosilybinin B. They found that significant variations in the ratios of the flavanolignan constituents among products and seeds, as well as variation between product and product label were detected.

In this work, it could be concluded that, the highest content of silychristin was found in samples collected from Romanian genotype cultivated in Egypt. Also, the highest content of silydianin was found in sample collected from desert road of Alexandria (violet flower). However, the highest content of silybinin (A, B) was found in sample collected from Kafr El-sheikh (violet flower). In this connection, it could be indicates that this is the first report about *silybum marianum* album in Egypt. According to the obtained results it could be observed that all genotypes tested showed fluctuations in the content of silychristin, silydianin, silybinin and total silymarin and this may be due to the environmental factors which affect the biosynthesis of the active ingredient besides the genetic make up. In this respect, many authors studied and developed methods for accurate analysis of the active ingredient in different medicinal plants. Recently, *Paramapojn and Gritsanapan (2008)* reported that HPLC method was developed and validated for quantitative determination of curcuminoid (curcumin, demethoxy-curcumin and bisdemethoxycurcumin) contents in extracts of the rhizomes of *Curcuma zedoaria* collected from ten locations in Thailand. The highest average total curcuminoid content in the powdered samples was found to be 2.50±0.52% w/w while the lowest content was 1.06±0.31% w/w.

In conclusion, it could be reported that the differences in geographical locations and the genetic variation between silybum genotypes have a great effect in their RAPD fingerprints and contents of silymarin.

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